invitrogen™

Champion™ pET Directional TOPO® Expression Kits

Five-minute, directional TOPO® Cloning of blunt-end PCR products into vectors for high-level, inducible expression in *E. coli*

Catalog nos. K100-01, K101-01, K102-01, K151-01, K200-01

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User Manual

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TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Step	Action
Design PCR Primers	• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with any epitope tags, if desired.
Amplify Your Gene of Interest	1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity and yield of your PCR product.
Perform the TOPO® Cloning Reaction	1. Set up the following TOPO® Cloning reaction. **For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector**. **Note:** If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in the TOPO® Cloning reaction. Fresh PCR product 0.5 to 4 µl Salt Solution 1 µl Sterile water add to a final volume of 5 µl TOPO® vector 1 µl Total volume 6 µl 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot® TOP10 chemically competent *E. coli*, below.
Transform TOP10 Chemically Competent *E. coli*	1. Add 3 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µl of room temperature S.O.C. medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 100-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 37-39 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following pET Directional TOPO® Expression kits listed below.

Kit	Quantity	Catalog no.
Champion™ pET100 Directional TOPO® Expression Kit	20 reactions	K100-01
Champion™ pET101 Directional TOPO® Expression Kit	20 reactions	K101-01
Champion™ pET102 Directional TOPO® Expression Kit	20 reactions	K102-01
Champion™ pET151 Directional TOPO® Expression Kit	20 reactions	K151-01
Champion™ pET200 Directional TOPO® Expression Kit	20 reactions	K200-01

Shipping/Storage

The Champion™ pET Directional TOPO® Expression Kits are shipped on dry ice. Each kit contains three boxes. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pET TOPO® Reagents	-20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	-80°C
3	BL21 Star™(DE3) One Shot® Chemically Competent *E. coli*	-80°C

Vectors and Primers

Each Champion™ pET Directional TOPO® Expression Kit contains a pET-TOPO® vector, an expression control, and primers for sequencing. The pET-TOPO® vector, expression control, and primers differ depending on the kit. The table below lists the vectors and primers supplied with each kit. For details on the amount of each component provided, see the next page.

pET TOPO® Kit	Catalog no.	TOPO® Vector	Expression Control	Primers
pET100	K100-01	pET100/D-TOPO®	pET100/D/*lacZ*	T7 T7 Reverse
pET101	K101-01	pET101/D-TOPO®	pET101/D/*lacZ*	T7 T7 Reverse
pET102	K102-01	pET102/D-TOPO®	pET102/D/*lacZ*	TrxFus Forward T7 Reverse
pET151	K151-01	pET151/D-TOPO®	pET151/D/*lacZ*	T7 T7 Reverse
pET200	K200-01	pET200/D-TOPO®	pET200/D/*lacZ*	T7 T7 Reverse

continued on next page

Kit Contents and Storage, continued

pET TOPO® Reagents

pET TOPO® Reagents (Box 1) are listed below. Each box includes PCR reagents and the appropriate vectors and primers. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pET TOPO® vector, TOPO®-adapted	15-20 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	--	1 ml
Forward Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl
Expression Control Plasmid	0.01 µg/µl in TE buffer, pH 8	10 µl

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Kit Contents and Storage, continued

Sequences of the Primers

Each Champion™ pET Directional TOPO® Expression Kit provides a forward and reverse sequencing primer to facilitate sequence analysis of your expression constructs (see the table on page vii for the specific primers included with each kit). The sequences of the forward and reverse primers are listed below. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
T7	5´-TAATACGACTCACTATAGGG-3´	327
TrxFus Forward	5´-TTCCTCGACGCTAACCTG-3´	371
T7 Reverse	5´-TAGTTATTGCTCAGCGGTGG-3´	325

One Shot® TOP10 Reagents

The table below lists the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is at least 1 x 10^9 cfu/µg DNA. **Store Box 2 at -80°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

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Kit Contents and Storage, continued

BL21 Star™(DE3) One Shot® Reagents

The table below describes the items included in the BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* kit (Box 3). Transformation efficiency is at least 1 x 10^8 cfu/µg DNA. **Store Box 3 at -80°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
BL21 Star™(DE3)	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

Use this *E. coli* strain for general cloning of blunt-end PCR products into the pET TOPO® vectors.

Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Genotype of BL21 Star™(DE3)

Use this *E. coli* strain for expression only. Do not use these cells to propagate or maintain your construct.

Genotype: F^- *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)

The DE3 designation means this strain contains the lambda DE3 lysogen which carries the gene for T7 RNA polymerase under the control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase.

The strain is an *E. coli* B/r strain and does not contain the *lon* protease. It also has a mutation in the outer membrane protease, OmpT. The lack of these two key proteases reduces degradation of heterologous proteins expressed in the strain.

The strain carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability (see page 6).

Accessory Products

Introduction

The products listed in this section are intended for use with the Champion™ pET Directional TOPO® Expression Kits. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 56).

Additional Products

Many of the reagents supplied in the Champion™ pET Directional TOPO® Expression Kits and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent *E. coli*	10 x 50 µl	C4040-10
	20 x 50 µl	C4040-03
BL21 Star™(DE3) One Shot® Chemically Competent *E. coli*	20 x 50 µl	C6010-03
BL21 Star™(DE3)pLysS One Shot® Chemically Competent *E. coli*	20 x 50 µl	C6020-03
BL21-AI™ One Shot® Chemically Competent *E. coli*	20 x 50 µl	C6070-03
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink™ Quick Gel Extraction Kit	50 reactions	K2100-12
Ampicillin	200 mg	11593-027
Kanamycin Sulfate	5 g	11815-024
	25 g	11815-032
Carbenicillin	5 g	10177-012
Isopropylthio-β-galactoside (IPTG)	1 g	15529-019
EKMax™	250 units	E180-01
	1000 units	E180-02
AcTEV Protease	1000 units	12575-015

continued on next page

Accessory Products, continued

Products to Detect Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The table below describes the products available from Invitrogen for detection of fusion proteins expressed using the appropriate pET TOPO® vector (see pages 11-15 for details about the N- and/or C-terminal tags present on each pET TOPO® vector).

The amount of antibody supplied is sufficient for 25 western blots.

Product	Mechanism of Detection	Catalog no.
Anti-Xpress™ Antibody	Detects 8 amino acid Xpress™ epitope: DLYDDDDK	R910-25
Anti-Xpress™-HRP Antibody		R911-25
Anti-HisG Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25
Anti-HisG-HRP Antibody		R941-25
Anti-HisG-AP Antibody		R942-25
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991): GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25
Anti-Thio™ Antibody	Detects His-Patch thioredoxin fusion proteins **Note:** The exact epitope detected by this antibody has not been mapped	R920-25

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Accessory Products, continued

Products to Purify Recombinant Fusion Proteins

If you clone your gene of interest in frame with a C-terminal or N-terminal peptide containing a polyhistidine (6xHis) tag, you may use Invitrogen's ProBond™ or Ni-NTA resins to purify your recombinant fusion protein. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
	150 ml	R801-15
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-Xpress™-HRP Antibody	1 kit	K851-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 kit	K854-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
	100 ml	R901-10
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Purification System with Anti-Xpress™-HRP Antibody	1 kit	K951-01
Ni-NTA Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K953-01
Ni-NTA Purification System with Anti-V5-HRP Antibody	1 kit	K954-01
Polypropylene Columns (empty)	50	R640-50

Introduction

Overview

Introduction

The Champion™ pET Directional TOPO® Expression Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for high-level, T7-regulated expression in *E. coli*. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

Depending on the vector chosen, the pET TOPO® vectors are available with:

- N-terminal or C-terminal peptide tags for production of recombinant fusion proteins that may be easily detected or purified
- Protease recognition site for removal of the N-terminal peptide tag from your recombinant fusion protein
- Antibiotic resistance marker for selection of transformants

See the table below for a list of the available pET TOPO® vectors and the fusion tag, cleavage site, and selection marker for each vector.

pET TOPO® vector	Fusion Peptide	Fusion Tag	Cleavage Site	Selection Marker
pET100/D-TOPO®	N-terminal	Xpress™, 6xHis	EK	Ampicillin
pET200/D-TOPO®				Kanamycin
pET101/D-TOPO®	C-terminal	V5, 6xHis	--	Ampicillin
pET102/D-TOPO®	N-terminal C-terminal	His-Patch thioredoxin V5, 6xHis	EK	Ampicillin
pET151/D-TOPO®	N-terminal	V5, 6xHis	TEV protease	Ampicillin

EK = enterokinase; TEV = tobacco etch virus

The Champion™ pET Expression System

The Champion™ pET Expression System is based on expression vectors originally developed by Studier and colleagues, and takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in *E. coli* from the T7 promoter (Rosenberg *et al.*, 1987; Studier & Moffatt, 1986; Studier *et al.*, 1990). For more information about the Champion™ pET Expression System, see page 4.

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Overview, continued

Features of the Champion™ pET Directional TOPO® Vectors

The pET TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for regulated expression in *E. coli*. Features of the vectors include:

- T7*lac* promoter for high-level, IPTG-inducible expression of the gene of interest in *E. coli* (Dubendorff & Studier, 1991; Studier *et al.*, 1990)
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- N- or C-terminal fusion tags for detection and purification of recombinant fusion proteins (choice of tag depends on the particular vector; see the previous page)
- Protease recognition site for cleavage of the fusion tag from the recombinant protein of interest (present on N-terminal fusion vectors)
- N-terminal His-Patch thioredoxin for increased translation efficiency and solubility of heterologous proteins (pET102/D-TOPO® only)
- *lacI* gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter in the pET TOPO® vector and from the *lacUV5* promoter in the *E. coli* host chromosome (see page 4 for more information)
- Antibiotic resistance marker for selection in *E. coli*
- pBR322 origin for low-copy replication and maintenance in *E. coli*

How Directional TOPO® Cloning Works

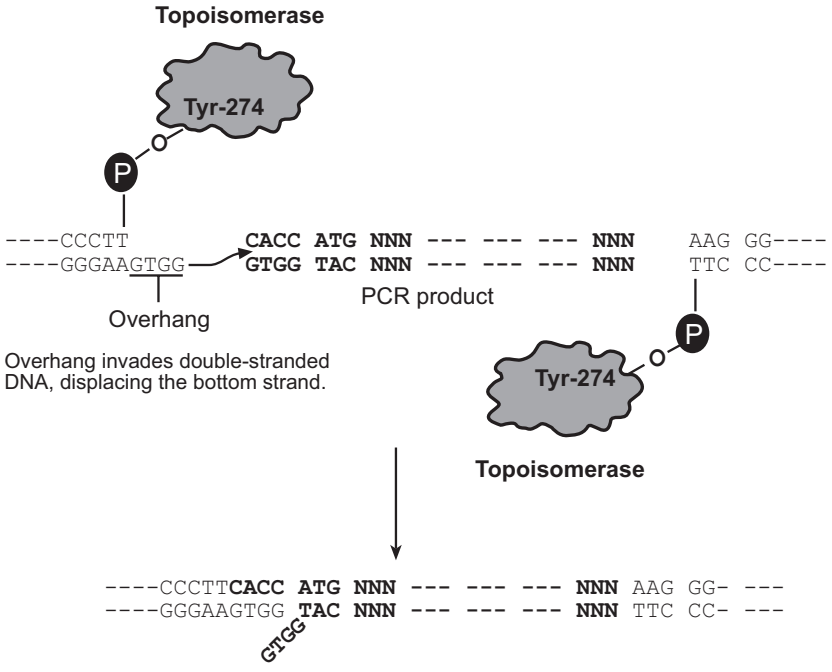
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng & Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



T7-Regulated Expression

The Basis of T7-Regulated Expression

The Champion™ pET Expression System uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. In the pET TOPO® vectors, expression of the gene of interest is controlled by a strong bacteriophage T7 promoter that has been modified to contain a *lac* operator sequence (see below). In bacteriophage T7, the T7 promoter drives expression of gene 10 (ϕ10). T7 RNA polymerase specifically recognizes this promoter. To express the gene of interest, it is necessary to deliver T7 RNA polymerase to the cells by inducing expression of the polymerase or infecting the cell with phage expressing the polymerase. In the Champion™ pET Directional TOPO® Expression System, T7 RNA polymerase is supplied by the BL21 Star™(DE3) host *E. coli* strain in a regulated manner (see below). When sufficient T7 RNA polymerase is produced, it binds to the T7 promoter and transcribes the gene of interest.

Regulating Expression of T7 RNA Polymerase

The BL21 Star™(DE3) *E. coli* strain is specifically included in each Champion™ pET Directional TOPO® Expression kit for expression of T7-regulated genes. This strain carries the DE3 bacteriophage lambda lysogen. This λDE3 lysogen contains a *lac* construct consisting of the following elements:

- the *lacI* gene encoding the lac repressor
- the T7 RNA polymerase gene under control of the *lacUV5* promoter
- a small portion of the *lacZ* gene.

This *lac* construct is inserted into the *int* gene such that it inactivates the *int* gene. Disruption of the *int* gene prevents excision of the phage (*i.e.* lysis) in the absence of helper phage. The *lac* repressor (encoded by *lacI*) represses expression of T7 RNA polymerase. Addition of the gratuitous inducer, isopropyl β-D-thiogalacto-side (IPTG) allows expression of T7 RNA polymerase from the *lacUV5* promoter.

The BL21 Star™(DE3) strain also contains other features which facilitate high-level expression of heterologous genes. For more information, see page 6.

T7*lac* Promoter

Studies have shown that there is always some basal expression of T7 RNA polymerase from the *lacUV5* promoter in λDE3 lysogens even in the absence of inducer (Studier & Moffatt, 1986). In general, this is not a problem, but if the gene of interest is toxic to the *E. coli* host, basal expression of the gene of interest may lead to plasmid instability and/or cell death.

To address this problem, the pET TOPO® vectors have been designed to contain a T7*lac* promoter to drive expression of the gene of interest. The T7*lac* promoter consists of a *lac* operator sequence placed downstream of the T7 promoter. The *lac* operator serves as a binding site for the lac repressor (encoded by the *lacI* gene) and functions to further repress T7 RNA polymerase-induced basal transcription of the gene of interest in BL21 Star™(DE3) cells.

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T7-Regulated Expression, continued

Expressing Toxic Genes

In some cases, the gene of interest is so toxic to BL21 Star™(DE3) cells that other *E. coli* host strains may be required for expression. For a discussion of other alternative strains that may be used, see page 6.

Using TOP10 Cells

One Shot® TOP10 competent *E. coli*, which do not contain T7 RNA polymerase, are included in each Champion™ pET Directional TOPO® Expression kit to provide a host for stable propagation and maintenance of recombinant plasmids. As mentioned on the previous page, the presence of T7 RNA polymerase, even at basal levels, can lead to expression of the desired gene even in the absence of inducer. If the gene of interest is toxic to the *E. coli* host, plasmid instability and/or cell death may result. **We recommend that you transform your TOPO® Cloning reaction into TOP10 cells for characterization of the construct, propagation, and maintenance.** When you are ready to perform an expression experiment, transform your construct into BL21 Star™(DE3) *E. coli*.

BL21 Star™ *E. coli* Strains

BL21 Star™ Strains

The BL21 Star™(DE3) *E. coli* strain is included in each Champion™ pET Directional TOPO® Expression Kit for use as a host for expression. Other BL21 Star™ strains are also available from Invitrogen (see below). In addition to the λDE3 lysogen which allows high-level expression of T7-regulated genes (see page 3), the BL21 Star™ strains also contain the *rne131* mutation. This particular mutation further enhances the expression capabilities of BL21 Star™.

rne131 Mutation

The *rne* gene encodes the RNase E enzyme, an essential, 1061 amino acid *E. coli* endonuclease which is involved in rRNA maturation and mRNA degradation as a component of a protein complex known as a "degradosome" (Grunberg-Manago, 1999; Lopez *et al.*, 1999). Various studies have shown that the N-terminal portion of RNase E (approximately 584 amino acids) is required for rRNA processing and cell growth while the C-terminal portion of the enzyme (approximately 477 amino acids) is required for mRNA degradation (Kido *et al.*, 1996; Lopez *et al.*, 1999). The *rne131* mutation (present in the BL21 Star™ strains) encodes a truncated RNase E which lacks the C-terminal 477 amino acids of the enzyme required for mRNA degradation (Kido *et al.*, 1996; Lopez *et al.*, 1999). Thus, mRNAs expressed in the RNase E-defective BL21 Star™ strains exhibit increased stability when compared to other BL21 strains. When heterologous genes are expressed in the BL21 Star™ strains from T7-based expression vectors, the yields of recombinant proteins generally increase.

BL21 Star™(DE3)pLysS Strain

If you discover that your gene is toxic to BL21 Star™(DE3) cells, you may want to perform your expression experiments in the BL21 Star™(DE3)pLysS strain (see page xi for ordering information). The BL21 Star™(DE3)pLysS strain contains the pLysS plasmid, which produces T7 lysozyme. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription. This activity results in reduced basal levels of T7 RNA polymerase, leading to reduced basal expression of T7-driven heterologous genes. For more information about BL21 Star™(DE3)pLysS, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 56).

Note

Note that while BL21 Star™(DE3)pLysS reduces basal expression from the gene of interest when compared to BL21 Star™(DE3), it also generally reduces the overall induced level of expression of recombinant protein.

Thioredoxin

Introduction

The pET102/D-TOPO® vector allows you to clone your gene of interest as a fusion to a mutated thioredoxin protein (His-Patch thioredoxin). For more information about thioredoxin and His-Patch thioredoxin, see below.

Thioredoxin

The 11.7 kDa thioredoxin protein is found in yeast, plants, and mammals, as well as in bacteria. It was originally isolated from *E. coli* as a hydrogen donor for ribonuclease reductase (see Holmgren, 1985 for a review). The gene has been completely sequenced (Wallace & Kushner, 1984). The protein has been crystallized and its three-dimensional structure determined (Katti *et al.*, 1990). When overexpressed in *E. coli*, thioredoxin is able to accumulate to approximately 40% of the total cellular protein and still remain soluble. When used as a fusion partner, thioredoxin can increase translation efficiency, and in some cases, solubility, of eukaryotic proteins expressed in *E. coli*.

Examples of eukaryotic proteins that have been produced as soluble C-terminal fusions to the thioredoxin protein in *E. coli* (LaVallie *et al.*, 1993) include:

- Murine interleukins 2, 4, and 5 and human interleukin-3
- Human macrophage colony stimulating factor
- Murine steel factor
- Murine leukemia inhibitory factor
- Human bone morphogenetic protein-2

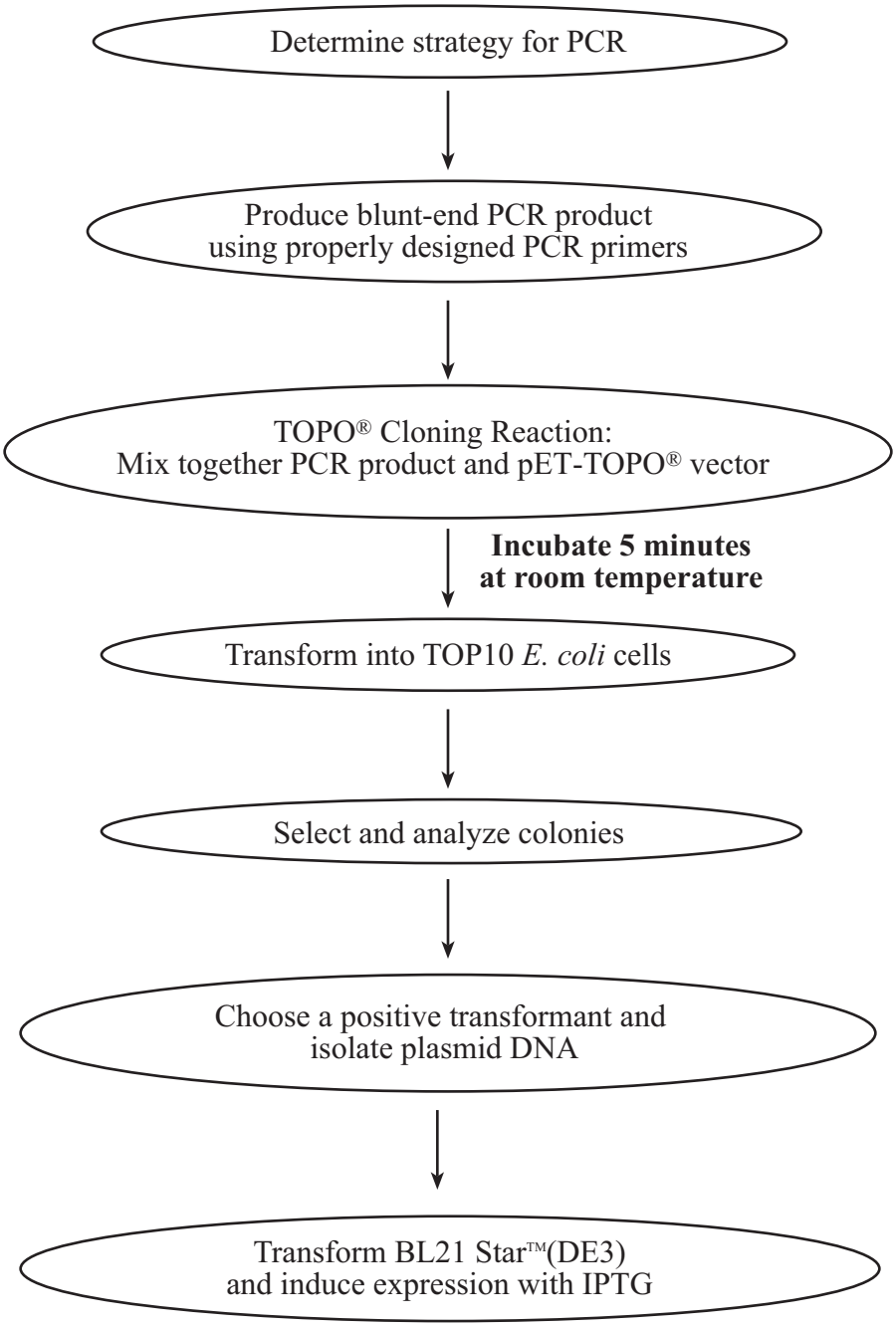
His-Patch Thioredoxin

The thioredoxin protein in pET102/D-TOPO® has been mutated to contain a metal binding domain, and is termed "His-Patch thioredoxin". To create a metal binding domain in the thioredoxin protein, the glutamate residues at position 32 and the glutamine residue at position 64 were mutated to histidine residues. When His-Patch thioredoxin folds, the histidines at positions 32 and 64 interact with a native histidine at position 8 to form a "patch". This histidine patch has been shown to have high affinity for divalent cations (Lu *et al.*, 1996). His-Patch thioredoxin (HP-thioredoxin) proteins can therefore be purified on metal chelating resins (*e.g.* ProBond™ or Ni-NTA).

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to clone and express your blunt-end PCR product.


Determine strategy for PCR
Produce blunt-end PCR product using properly designed PCR primers
TOPO® Cloning Reaction: Mix together PCR product and pET-TOPO® vector
Incubate 5 minutes at room temperature
Transform into TOP10 E. coli cells
Select and analyze colonies
Choose a positive transformant and isolate plasmid DNA
Transform BL21 Star™(DE3) and induce expression with IPTG

Designing PCR Primers

Basic Requirements

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pET TOPO® vector you are using, consider the following when designing your PCR primers:

- Sequences required to facilitate directional cloning (see below)
- Whether or not you wish to clone your PCR product in frame with the appropriate N-terminal and/or C-terminal peptide tag (see pages 11-15 for information about each pET TOPO® vector)

General Requirements for the Forward Primer

To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pET TOPO® vector.

For example, below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer:

DNA sequence: `5′-ATG GGA TCT GAT AAA`
Proposed Forward PCR primer: `5′-C ACC ATG GGA TCT GAT AAA`

See pages 11-15 for other factors to consider when designing the forward primer.

General Requirements for the Reverse Primer

In general, design the reverse PCR primer to allow you to clone your PCR product in frame with any C-terminal tag, if desired. **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 75%, and may increase the chances of your ORF cloning in the opposite orientation.** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch, but this has not been tested thoroughly.

Example: Below is the sequence of the C-terminus of a theoretical protein. You want to clone in frame with the C-terminal tag. The stop codon is underlined.

DNA sequence: `AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′`

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the overhang sequence. As a result, the reverse primer will be complementary to the overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: `AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′`
Proposed Reverse PCR primer sequence: `TG AGC TGC TGC CAC-5′`

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine or alanine.

continued on next page

Basic Requirements, continued

Important

- Remember that the pET TOPO® vectors accept blunt-end PCR products. Refer to pages 11-15 for a discussion of specific factors to consider when designing PCR primers for cloning into each pET TOPO® vector.
- Do not add 5´ phosphates to your primers for PCR. This will prevent ligation into the pET TOPO® vectors.
- We recommend gel-purifying your oligonucleotides, especially if they are long (> 30 nucleotides).

Example of Primer Design

The example below uses a theoretical protein and is for illustration purposes only. In this case, PCR primers are designed to allow cloning of the PCR product into pET101/D-TOPO®. In this example, the N-terminus of the protein is encoded by:

5′-ATGGCCCCCCCGACCGATGTCAGCCTGGGGGACGAA…

1. Design the forward PCR primer to be:

   5′-CACCATGGCCCCCCCGACCGAT-3′

2. For the reverse primer, analyze the C-terminus of the protein. The stop codon is underlined (see the top strand below).

   …GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′
   …CGC CAA TTC AGC CTC GTG AGC TGC TGA CGT ATC-5′

3. To fuse the ORF in frame with the V5 epitope and 6xHis tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream (underlined sequence in the bottom strand above). The reverse primer will be:

   5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

4. This will amplify the C-terminus without the stop codon and allow you to clone the ORF in frame with the V5 epitope and 6xHis tag.

   If you don't want the V5 epitope and 6xHis tag, simply begin with the stop codon:

   5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Specific Requirements for Cloning into pET100/D-TOPO® and pET200/D-TOPO®

Introduction

pET100/D-TOPO® and pET200/D-TOPO® allow expression of recombinant protein with an N-terminal tag containing the Xpress™ epitope and a 6xHis tag. The N-terminal tag also includes an enterokinase (EK) recognition site to enable removal of the tag after protein purification using enterokinase (*e.g.* EKMax™).

Additional Cloning Considerations

In addition to the guidelines on page 9, consider the following when designing PCR primers to clone your DNA into pET100/D-TOPO® or pET200/D-TOPO®.

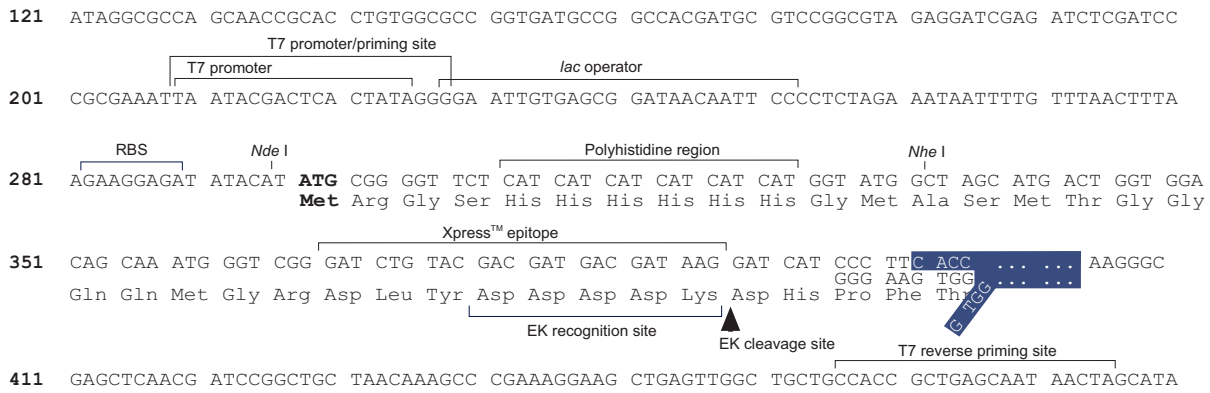
Be sure to include a stop codon in the reverse primer or design the reverse primer to hybridize downstream of the native stop codon.

If you wish to...	Then...
include the Xpress™ epitope and 6xHis tag	design the forward PCR primer to place the gene of interest in frame with the N-terminal tag. Note that: • a ribosome binding site (RBS) is included upstream of the initiation ATG in the N-terminal tag to ensure optimal spacing for proper translation • at least five nonnative amino acids will be present between the EK cleavage site and the start of your gene
express your protein with a native N-terminus, *i.e.* without the N-terminal peptide	design the forward PCR primer to include the following: • a stop codon to terminate the N-terminal peptide • a second ribosome binding site (AGGAGG) 9-10 base pairs 5′ of the initiation ATG codon of your protein

Note: The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a complete codon.

TOPO® Cloning Site of pET100/D-TOPO® and pET200/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pET100/D-TOPO® or pET200/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. **The sequence of each vector is available for downloading from our Web site or from Technical Service (see page 56).** For more information about pET100/D-TOPO® or pET200/D-TOPO®, see pages 42-43.


121 ATAGGCGCCA GCAACCGCAC CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC
T7 promoter/priming site
T7 promoter
lac operator
201 CGCGAAATTA ATACGACTCA CTATAGGGGA ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA
RBS
Nde I
Polyhistidine region
Nhe I
281 AGAAGGAGAT ATACAT ATG CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA
Met Arg Gly Ser His His His His His His Gly Met Ala Ser Met Thr Gly Gly
Xpress™ epitope
351 CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CAT CCC TTC ACC ... ... AAGGGC
GGG AAG TGG ... ...
Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp His Pro Phe Th
G TGG
EK recognition site
EK cleavage site
T7 reverse priming site
411 GAGCTCAACG ATCCGGCTGC TAACAAAGCC CGAAAGGAAG CTGAGTTGGC TGCTGCCACC GCTGAGCAAT AACTAGCATA

Specific Requirements for Cloning into pET101/D-TOPO®

Additional Cloning Considerations

pET101/D-TOPO® allows expression of recombinant protein with a native N-terminus and a C-terminal fusion tag. In addition to the guidelines on page 9, consider the following when designing PCR primers to clone your DNA into pET101/D-TOPO®.

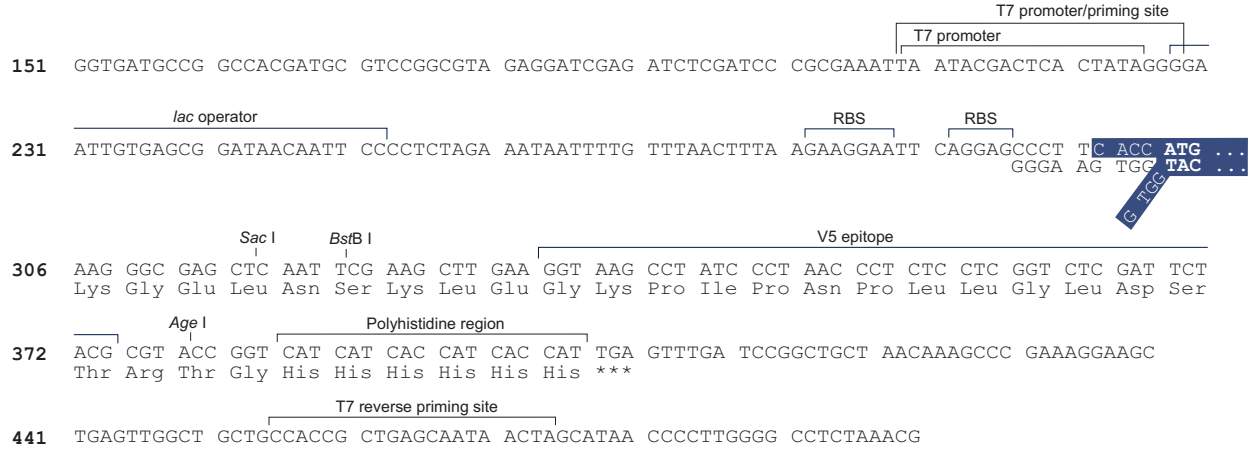
For maximal expression of native protein, the forward PCR primer should be designed to place the initial ATG codon of the desired protein approximately 9 to 10 base pairs from the ribosome binding site (Gold, 1988; Miller, 1992). This will ensure the optimal spacing for proper translation.

If you wish to...	Then...
express your protein with a native N-terminus using the vector encoded ribosome binding site	design the forward PCR primer such that the initial ATG codon of your protein directly follows the 5´ CACC overhang.
include the C-terminal V5 epitope and 6xHis tag	design the reverse PCR primer to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag.
not include the C-terminal V5 epitope and 6xHis tag	include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site.

Note: The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a complete codon.

TOPO® Cloning Site of pET101/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pET101/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. **The sequence of the vector is available for downloading from our Web site or from Technical Service (see page 56).** For more information about pET101/D-TOPO®, see pages 44-45.


T7 promoter/priming site
T7 promoter
151 GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC CGCGAAATTA ATACGACTCA CTATAGGGGA
lac operator
RBS
RBS
231 ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA AGAAGGAATT CAGGAGCCCT TC ACC ATG ...
GGGA AG TGG TAC ...
G TGG
Sac I
BstB I
V5 epitope
306 AAG GGC GAG CTC AAT TCG AAG CTT GAA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT
Lys Gly Glu Leu Asn Ser Lys Leu Glu Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser
Age I
Polyhistidine region
372 ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTGA TCCGGCTGCT AACAAAGCCC GAAAGGAAGC
Thr Arg Thr Gly His His His His His His ***
T7 reverse priming site
441 TGAGTTGGCT GCTGCCACCG CTGAGCAATA ACTAGCATAA CCCCTTGGGG CCTCTAAACG

Specific Requirements for Cloning into pET102/D-TOPO®

Specific Features for Expression in pET102/D-TOPO®

pET102/D-TOPO® is designed with some specific features to facilitate expression. They are:

- The initiation ATG is correctly spaced from the optimized ribosome binding site (RBS) to ensure optimal translation
- HP-thioredoxin acts to increase translation efficiency and in some cases, solubility
- HP-thioredoxin can be removed after protein purification using enterokinase (*e.g.* EKMax™, Catalog no. E180-01).

Additional Cloning Considerations

In addition to the guidelines on page 9, you should consider the following when designing PCR primers to clone your DNA into pET102/D-TOPO®. Use the diagram on the next page to help you design your PCR primers.

If you wish to...	Then...
clone in frame with thioredoxin	design the forward PCR primer to ensure that your protein is in frame with the N-terminal leader peptide.
include the C-terminal V5 epitope and 6xHis tag	design the reverse PCR primer to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag.
not include the C-terminal V5 epitope and 6xHis tag	include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site.

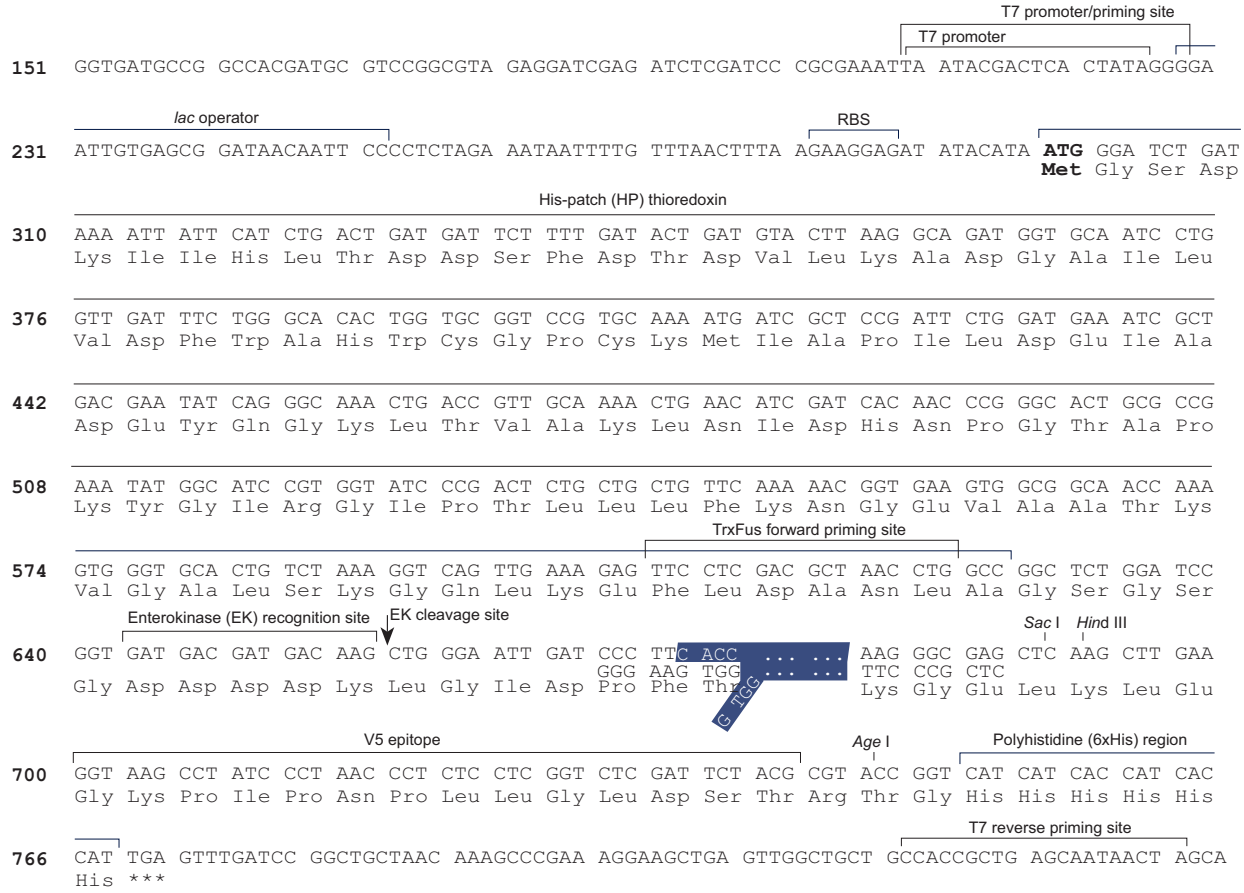
Note: The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a complete codon.

continued on next page

Specific Requirements for Cloning into pET102/D-TOPO®, continued

TOPO® Cloning Site of pET102/D-TOPO®

Use the diagram below to help you design appropriate PCR primers to clone your PCR product into pET102/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. **The complete sequence of the vector is available for downloading from our Web site or from Technical Service (see page 56).** For more information about pET102/D-TOPO®, refer to the **Appendix**, pages 46-47.


T7 promoter/priming site
T7 promoter
151 GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC CGCGAAATTA ATACGACTCA CTATAGGGGA
lac operator
RBS
231 ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA AGAAGGAGAT ATACATA ATG GGA TCT GAT
Met Gly Ser Asp
His-patch (HP) thioredoxin
310 AAA ATT ATT CAT CTG ACT GAT GAT TCT TTT GAT ACT GAT GTA CTT AAG GCA GAT GGT GCA ATC CTG
Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly Ala Ile Leu
376 GTT GAT TTC TGG GCA CAC TGG TGC GGT CCG TGC AAA ATG ATC GCT CCG ATT CTG GAT GAA ATC GCT
Val Asp Phe Trp Ala His Trp Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala
442 GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAC AAC CCG GGC ACT GCG CCG
Asp Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp His Asn Pro Gly Thr Ala Pro
508 AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA
Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys
TrxFus forward priming site
574 GTG GGT GCA CTG TCT AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCC GGC TCT GGA TCC
Val Gly Ala Leu Ser Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala Gly Ser Gly Ser
Enterokinase (EK) recognition site
EK cleavage site
Sac I
Hind III
640 GGT GAT GAC GAT GAC AAG CTG GGA ATT GAT CCC TTC ACC ... ... AAG GGC GAG CTC AAG CTT GAA
GGG AAG TGG ... ... TTC CCG CTC
Gly Asp Asp Asp Asp Lys Leu Gly Ile Asp Pro Phe Thr Lys Gly Glu Leu Lys Leu Glu
G TGG
V5 epitope
Age I
Polyhistidine (6xHis) region
700 GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC
Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His
T7 reverse priming site
766 CAT TGA GTTTGATCC GGCTGCTAAC AAAGCCCGAA AGGAAGCTGA GTTGGCTGCT GCCACCGCTG AGCAATAACT AGCA
His ***

Specific Requirements for Cloning into pET151/D-TOPO®

Introduction

pET151/D-TOPO® allows expression of recombinant protein with an N-terminal tag containing the V5 epitope and a 6xHis tag. The N-terminal tag also includes a TEV protease cleavage site to enable removal of the tag after protein purification using TEV protease.

Additional Cloning Considerations

In addition to the guidelines on page 9, consider the following when designing PCR primers to clone your DNA into pET151/D-TOPO®.

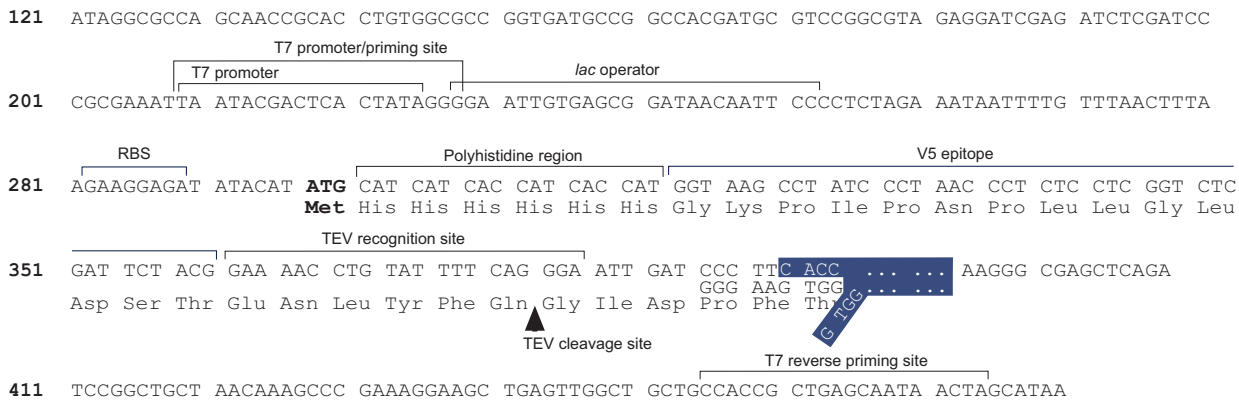
Be sure to include a stop codon in the reverse primer or design the reverse primer to hybridize downstream of the native stop codon.

If you wish to...	Then...
include the V5 epitope and 6xHis tag	design the forward PCR primer to place the gene of interest in frame with the N-terminal tag. Note that: a ribosome binding site (RBS) is included upstream of the initiation ATG in the N-terminal tag to ensure optimal spacing for proper translation at least six nonnative amino acids will be present between the TEV cleavage site and the start of your gene
express your protein with a native N-terminus, *i.e.* without the N-terminal peptide	design the forward PCR primer to include the following: a stop codon to terminate the N-terminal peptide a second ribosome binding site (AGGAGG) 9-10 base pairs 5′ of the initiation ATG codon of your protein

Note: The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a complete codon.

TOPO® Cloning Site of pET151/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pET151/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. **The sequence of pET151/D-TOPO® is available for downloading from our Web site or from Technical Service (see page 56).** For more information about pET151/D-TOPO®, see pages 48-49.


121 ATAGGCGCCA GCAACCGCAC CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC
T7 promoter/priming site
T7 promoter
lac operator
201 CGCGAAATTA ATACGACTCA CTATAGGGGA ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA
RBS
Polyhistidine region
V5 epitope
281 AGAAGGAGAT ATACAT ATG CAT CAT CAC CAT CAC CAT GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC
Met His His His His His His Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu
TEV recognition site
351 GAT TCT ACG GAA AAC CTG TAT TTT CAG GGA ATT GAT CCC TTC ACC ... ... AAGGG CGAGCTCAGA
GGG AAG TGG ... ...
Asp Ser Thr Glu Asn Leu Tyr Phe Gln Gly Ile Asp Pro Phe Th
G TGG
TEV cleavage site
T7 reverse priming site
411 TCCGGCTGCT AACAAAGCCC GAAAGGAAGC TGAGTTGGCT GCTGCCACCG CTGAGCAATA ACTAGCATAA

Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Needed

You should have the following materials on hand before beginning.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers for PCR product

Producing PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below:

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at -20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations for optimizing your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see page 40s).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

TOPO® Cloning Reaction and Transformation

Setting Up the TOPO® Cloning Reaction

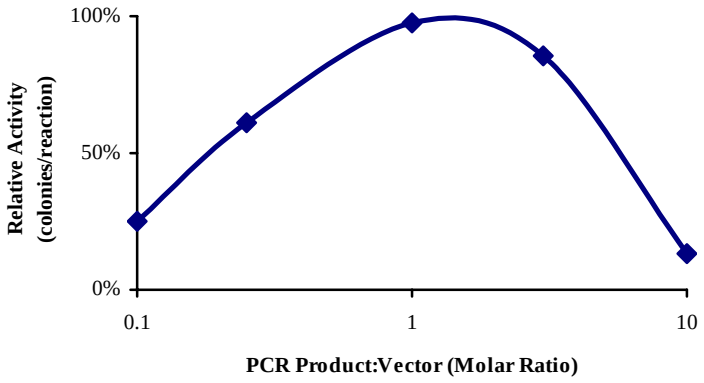
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pET TOPO® vector and transform the recombinant vector into One Shot® TOP10 *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read the this section and the section entitled **Transforming Competent Cells** before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 37-39 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pET TOPO® vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Setting Up the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page xi for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Total Volume	**6 µl**	**6 µl**

*Store all reagents at -20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pET TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells. Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

To maintain the stability of your construct, we recommend that you transform your TOPO® Cloning reaction into TOP10 cells and characterize transformants in TOP10 before proceeding to expression studies using BL21 Star™(DE3). Expression of T7 RNA polymerase in BL21 Star™(DE3) may be leaky and may lead to rearrangement or loss of your plasmid.

Materials Supplied by the User

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing the appropriate antibiotic for selection (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation, reducing the number of colonies to be analyzed. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing the appropriate antibiotic (*i.e.* 50-100 µg/ml ampicillin or 50-100 µg/ml kanamycin, as appropriate) at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

Important

The number of colonies obtained after transforming the pET TOPO® vectors into One Shot® TOP10 cells is generally lower when compared to the number of colonies obtained after transforming other prokaryotic TOPO® vectors (*e.g.* pCR®T7 TOPO®, pBAD/Thio-TOPO®). This is due to the following:

- Directional TOPO® Cloning generally yields 2 to 5-fold fewer colonies than traditional bidirectional TOPO TA Cloning®
- Transforming low-copy number TOPO® plasmids generally yields 2 to 5-fold fewer colonies than transforming high-copy number TOPO® plasmids

If you have TOPO® Cloned previously, note that we have slightly modified the One Shot® TOP10 transformation protocols (see below and the next page) to address this issue. Briefly, we recommend the following:

- Increase the amount of TOPO® Cloning reaction that you transform into TOP10 cells (use 3 µl) **and**
- Increase the amount of transformed cells that you plate (use 100-200 µl for chemically competent cells and 50-100 µl for electrocompetent cells)

Example: When directionally TOPO® Cloning a 750 bp test insert into any of the pET TOPO® vectors, we generally obtain 500-1500 total colonies. Although fewer total colonies are obtained, greater than 90% of the colonies will contain plasmid with your PCR insert in the correct orientation.

One Shot® TOP10 Chemical Transformation Protocol

1. Add 3 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 18 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 100-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Positive Clones**, page 22).

   Note: If you see few transformants, refer to the **Troubleshooting** section, page 34 for tips to optimize your TOPO® Cloning and transformation reactions.

continued on next page

Transforming Competent Cells, continued

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 3 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 18 into 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the electrocompetent cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and an electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 ml snap-cap tube (i.e. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance marker.
5. Spread 50-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Positive Clones**, page 22).

   Note: If you see few transformants, refer to the **Troubleshooting** section, page 34 for tips to optimize your TOPO® Cloning and transformation reactions.

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or S.O.B. medium containing the appropriate antibiotic.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

   Note: Since the pET TOPO® vectors are low-copy number plasmids, you may need to increase the amount of bacterial culture to obtain enough plasmid DNA for sequencing or analysis purposes. Use extra care during purification to obtain plasmid DNA of sufficiently pure quality for sequencing (see below).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

We recommend sequencing your construct to confirm that your gene is in frame with the appropriate N-terminal or C-terminal fusion tag, if desired. The table below lists the primers included in each kit to help you sequence your insert.

Vector	Forward Primer	Reverse Primer
pET100/D-TOPO®	T7	T7 Reverse
pET101/D-TOPO®	T7	T7 Reverse
pET102/D-TOPO®	TrxFus Forward	T7 Reverse
pET151/D-TOPO®	T7	T7 Reverse
pET200/D-TOPO®	T7	T7 Reverse

Important

If you download the sequence from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

continued on next page

Analyzing Transformants, continued

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the Forward sequencing primer or the Reverse sequencing primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 37-39. These reactions will help you troubleshoot your experiment. Refer to the **Troubleshooting** section, page 34 for additional tips.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing the appropriate antibiotic.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing the appropriate antibiotic.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Expression and Purification

General Guidelines for Expression

Introduction

BL21 Star™(DE3) One Shot® *E. coli* (Box 3) are included with each Champion™ pET Directional TOPO® Expression Kit for use as the host for expression. You will need pure plasmid DNA of your pET TOPO® construct to transform into BL21 Star™(DE3) for expression studies. Since each recombinant protein has different characteristics that may affect optimal expression, we recommend performing a time course of expression to determine the best conditions for expression of your protein. Each Champion™ pET Directional TOPO® Expression Kit also includes the appropriate pET TOPO® vector containing the *lacZ* gene for use as a positive expression control (see below).

BL21 Star™ Strains

The BL21 Star™(DE3) *E. coli* strain is specifically designed for expression of genes regulated by the T7 promoter. Each time you perform an expression experiment, you will transform your plasmid into BL21 Star™(DE3). **Do not use this strain for propagation and maintenance of your plasmid. Use TOP10 instead.** Basal level expression of T7 polymerase, particularly in BL21 Star™(DE3) cells, may lead to plasmid instability if your gene of interest is toxic to *E. coli*.

Note: If you are expressing a highly toxic gene, the BL21 Star™(DE3)pLysS strain is also available from Invitrogen for expression purposes. The BL21 Star™(DE3)pLysS strain contains the pLysS plasmid to further reduce basal level expression of the gene of interest. For more information, see page 6.

Positive Controls

Each Champion™ pET Directional TOPO® Expression Kit includes a positive control vector for use as an expression control (see the table below). In each case, the gene encoding β-galactosidase is directionally TOPO® Cloned into the appropriate pET TOPO® vector (see pages 50-53 for details). Transform 10 ng of each plasmid into BL21 Star™(DE3) cells using the procedure on page 26.

Kit	Positive Control
Champion™ pET100 Directional TOPO® Expression Kit	pET100/D/*lacZ*
Champion™ pET101 Directional TOPO® Expression Kit	pET101/D/*lacZ*
Champion™ pET102 Directional TOPO® Expression Kit	pET102/D/*lacZ*
Champion™ pET151 Directional TOPO® Expression Kit	pET151/D/*lacZ*
Champion™ pET200 Directional TOPO® Expression Kit	pET200/D/*lacZ*

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General Guidelines for Expression, continued

Basic Strategy

The basic steps needed to induce expression of your gene in BL21 Star™(DE3) *E. coli* are outlined below.

1. Isolate plasmid DNA using standard procedures and transform your construct and the positive control separately into BL21 Star™(DE3) One Shot® cells.
2. Grow the transformants and induce expression with IPTG over several hours. Take several time points to determine the optimal time of expression.
3. Optimize expression to maximize the yield of protein.

Plasmid Preparation

You may prepare plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) for isolation of pure plasmid DNA. Note that since you are purifying a low-copy number plasmid, you may need to increase the amount of bacterial culture that you use to prepare your plasmid construct.

Ampicillin Selection

For pET TOPO® vectors containing the ampicillin resistance gene, ampicillin generally works well for selection of transformants and expression experiments. However, if you find that your expression levels are low, you may want to use carbenicillin instead. The resistance gene for ampicillin encodes the protein, β-lactamase. β-lactamase is secreted into the medium where it hydrolyzes ampicillin, inactivating the antibiotic. Since β-lactamase is catalytic, ampicillin is rapidly removed from the medium, resulting in non-selective conditions. If your plasmid is unstable, this may result in the loss of plasmid and low expression levels.

Using Carbenicillin

Carbenicillin is generally more stable than ampicillin, and studies have shown that using carbenicillin in place of ampicillin may help to increase expression levels by preventing loss of the pET TOPO® plasmid. If you wish to use carbenicillin, perform your transformation and expression experiments in LB containing 50 μg/ml carbenicillin.

Note: If your gene of interest is highly toxic, increasing the concentration of carbenicillin used from 50 μg/ml to 200 μg/ml may help to increase expression levels.

Note

Note that cyclic AMP-mediated derepression of the *lacUV5* promoter in λDE3 lysogens can result in an increase in basal expression of T7 RNA polymerase. If you are expressing an extremely toxic gene, the pET construct may be unstable in BL21 Star™(DE3) cells. Adding 1% glucose to the bacterial culture medium may help to repress basal expression of T7 RNA polymerase and stabilize your pET construct.

Expressing the PCR Product

Materials to Have on Hand

Be sure to have the following solutions and equipment on hand before starting your expression experiment:

- Your pET TOPO® expression construct (>10 µg/ml)
- The appropriate pET/D/*lacZ* positive control plasmid
- BL21 Star™(DE3) One Shot® cells (Box 3 supplied with the kit)
- S.O.B. or LB containing the appropriate antibiotic for selection (plus 1% glucose, if desired)
- 37°C incubator (shaking and nonshaking)
- 42°C water bath
- 1 M isopropyl β-D-thiogalactoside (IPTG; Invitrogen, Catalog no. 15529-019)
- Liquid nitrogen

Transforming BL21 Star™(DE3) One Shot® Cells

To transform your construct or the positive control (10 ng each) into BL21 Star™(DE3) One Shot® cells, follow the instructions below. You will need one vial of cells per transformation.

Note: You will not plate the transformation reaction, but inoculate it into medium for growth and subsequent expression.

1. Thaw on ice, one vial of BL21 Star™(DE3) One Shot® cells per transformation.
2. Add 5-10 ng plasmid DNA in a 1 to 5 µl volume into each vial of BL21 Star™(DE3) One Shot® cells and mix by stirring gently with the pipette tip. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 250 µl of room temperature S.O.C. medium.
7. Cap the tube tightly, tape the tube on its side (for better aeration), and incubate at 37°C for 30 minutes with shaking (200 rpm).
8. Add the **entire** transformation reaction to 10 ml of LB containing the appropriate antibiotic (and 1% glucose, if desired).
9. Grow overnight at 37°C with shaking. Proceed to **Pilot Expression**, next page.

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Expressing the PCR Product, continued

Pilot Expression

1. Inoculate 10 ml of LB containing the appropriate antibiotic (and 1% glucose, if desired) with 500 µl of the overnight culture from Step 8, previous page.
2. Grow two hours at 37°C with shaking. OD_{600} should be about 0.5-0.8 (mid-log).
3. Split the culture into two 5 ml cultures. Add IPTG to a final concentration of 0.5-1 mM to one of the cultures. You will now have two cultures: one induced, one uninduced.
4. Remove a 500 µl aliquot from **each** culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
5. Freeze the cell pellets at -20°C. These are the zero time point samples.
6. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 4 to 6 hours.
7. For each time point, remove 500 µl from the induced and uninduced cultures and process as described in Steps 4 and 5. Proceed to **Analyzing Samples**, next page.

Analyzing Samples

Materials to Have on Hand

Have the following materials on hand before starting:

- Lysis Buffer (see page 55 for recipe)
- 1X and 2X SDS-PAGE sample buffer (see page 55 for recipes)
- Reagents and apparatus to perform SDS-PAGE electrophoresis
- Boiling water bath

Preparing Samples

Once you have finished your pilot expression, you are ready to analyze the samples you have collected. Before starting, prepare SDS-PAGE gels or use one of the pre-cast polyacrylamide gels available from Invitrogen (see below).

Note: If you wish to analyze your samples for soluble protein, see below.

1. Thaw the samples (from Pilot Expression, Steps 5 and 7, previous page) and resuspend each cell pellet in 80 µl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5-10 µl of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing them at -20°C.

Preparing Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each cell pellet in 500 µl of Lysis Buffer (see **Recipes**, page 55).
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times.

   Note: To facilitate lysis, you may need to add lysozyme or sonicate the cells.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer and boil for 5 minutes.
5. Add 500 µl of 1X SDS-PAGE sample buffer to the pellets from Step 3 and boil 5 minutes.
6. Load 10 µl of the supernatant sample and 5 µl of the pellet sample onto an SDS-PAGE gel and electrophorese.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 56).

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Analyzing Samples, continued

Analyzing Samples

To determine the success of your expression experiment, you may want to perform the following types of analyses:

1. Stain the polyacrylamide gel with Coomassie blue and look for a band of increasing intensity in the expected size range for the recombinant protein. Use the uninduced culture as a negative control.
2. Perform a western blot to confirm that the overexpressed band is your desired protein (see below).
3. Use the positive control to confirm that growth and induction were performed properly. The table below lists the size of the β-galactosidase fusion protein expressed from each positive control vector.

   Note: β-galactosidase Antiserum is available from Invitrogen (Catalog no. R901-25) to detect β-galactosidase fusion proteins by western blot.

Vector	Size of the Control Protein
pET100/D/*lacZ*	121 kDa
pET101/D/*lacZ*	120 kDa
pET102/D/*lacZ*	133 kDa
pET151/D/*lacZ*	120 kDa
pET200/D/*lacZ*	121 kDa

Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use:

- Antibodies against the appropriate epitope available from Invitrogen (see page xii for ordering information)
- An antibody to your protein of interest

In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing an Xpress™, HisG, V5, or C-terminal 6xHis epitope. The WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescence methods. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 56).

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Analyzing Samples, continued

Note

Expression of your protein with the N- and/or C-terminal tags will increase the size of your recombinant protein. The table below lists the increase in the molecular weight of your recombinant fusion protein that you should expect from the particular N- or C-terminal fusion tag in each pET TOPO® vector. Be sure to account for any additional amino acids between the fusion tag and the start of your protein.

Vector	Fusion Tag	Expected Size Increase (kDa)
pET100/D-TOPO®	N-terminal	3 kDa
pET101/D-TOPO®	C-terminal	3 kDa
pET102/D-TOPO®	N-terminal	13 kDa
	C-terminal	3 kDa
pET151/D-TOPO®	N-terminal	4 kDa
pET200/D-TOPO®	N-terminal	3 kDa

The Next Step

If you are satisfied with expression of your gene of interest, proceed to **Purifying the Recombinant Fusion Protein**, page 31.

If you have trouble expressing your protein or wish to optimize expression, refer to the **Troubleshooting** section, page 37.

Purifying the Recombinant Fusion Protein

Introduction

The presence of the N-terminal or C-terminal polyhistidine (6xHis) tag in all pET TOPO® vectors allows purification of your recombinant fusion protein with a metal-chelating resin such as ProBond™ or Ni-NTA.

ProBond™ and Ni-NTA

ProBond™ and Ni-NTA are nickel-charged agarose resins that can be used for affinity purification of fusion proteins containing the 6xHis tag and/or the HP-thioredoxin protein. Proteins bound to the resin may be eluted with either low pH buffer or competition with imidazole or histidine.

- To scale up your pilot expression for purification, see below.
- To purify your fusion protein using ProBond™ or Ni-NTA, refer to the manual included with each product. You may download the manuals from our Web site (www.invitrogen.com).
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Important

Note that under denaturing conditions, the Ni^{2+} binding site encoded by the histidine "patch" in pET102/D-TOPO® will be destroyed because the HP-thioredoxin protein will be denatured. The binding of nickel ion to the 6xHis tag is not affected by denaturing conditions.

Scaling-up Expression for Purification

We generally scale-up expression to a 50 ml bacterial culture for purification using a 2 ml ProBond™ or Ni-NTA column. Depending on the expression level of your recombinant fusion protein, you may need to adjust the culture volume to bind the maximum amount of recombinant fusion protein to your column.

To grow and induce a 50 ml bacterial culture:

1. Inoculate 10 ml of S.O.B. or LB containing the appropriate antibiotic with 500 µl of the culture of the transformation reaction (see page 26, step 9).
2. Grow overnight at 37°C with shaking (225-250 rpm) to OD_{600} = 1-2.
3. The next day, inoculate 50 ml of S.O.B. or LB containing the appropriate antibiotic with 1 ml of the overnight culture.

   Note: You can scale up further and inoculate all of the 10 ml overnight culture into 500 ml of medium, but you will need to adjust the bed volume of your ProBond™ or Ni-NTA column accordingly.
4. Grow the culture at 37°C with shaking (225-250 rpm) to an OD_{600} = ~0.5 (2-3 hours). The cells should be in mid-log phase.
5. Add 0.5-1 mM IPTG to induce expression.
6. Grow at 37°C with shaking until the optimal time point determined by the pilot expression is reached. Harvest the cells by centrifugation (3000 x g for 10 minutes at +4°C).
7. Proceed to purification or store the cells at -80°C for future use.

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Purifying the Recombinant Fusion Protein, continued

Additional Purification Steps

There may be cases when your specific fusion protein may not be completely purified by metal affinity chromatography. Other protein purification techniques may be utilized in conjunction with ProBond™ or Ni-NTA to purify the fusion protein (see Deutscher, 1990 for more information).

Removing N-terminal Fusion Tags

Introduction

If you have expressed your recombinant fusion protein from any N-terminal fusion pET TOPO® vector, the presence of either the enterokinase (EK) or TEV recognition site in the vector allows removal of the N-terminal fusion tag from the recombinant fusion protein after purification using enterokinase or TEV protease, as appropriate. General guidelines are provided below.

Removal of the N-terminal Fusion Tag Using Enterokinase

If you have expressed your recombinant fusion protein from pET TOPO® vectors containing the EK recognition site (see below), you may use a recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) available from Invitrogen (Catalog no. E180-01) to remove the N-terminal fusion tag. Instructions for digestion are included with the product. For more information, contact Technical Service (see page 56). To remove EKMax™ from the digest, EK-Away™ (Catalog no. R180-01) is also available.

Note: After digestion with enterokinase, a number of vector-encoded amino acids will remain at the N-terminus of your protein (see table below).

Vector	Number of Amino Acids Remaining After EK Cleavage
pET100/D-TOPO®	5
pET102/D-TOPO®	7
pET200/D-TOPO®	5

Removal of the N-terminal Fusion Tag Using TEV Protease

If you have expressed your recombinant fusion protein from pET151/D-TOPO®, you may use recombinant TEV Protease available from Invitrogen (Catalog no. 12575-015) to remove the N-terminal fusion tag. Instructions for digestion are included with the product. For more information, contact Technical Service (see page 56).

Note: After digestion with enterokinase, six vector-encoded amino acids will remain at the N-terminus of your protein.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 37-39) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	Dilute the PCR product. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR product too dilute	• Concentrate the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify your PCR product.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.

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Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
Large number of incorrect inserts cloned	PCR cloning artifacts	Gel-purify your PCR product to remove primer-dimers and smaller PCR products. Optimize your PCR. Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	Make sure that the forward and reverse PCR primers are designed correctly.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	One Shot® transformation protocol not followed correctly	Follow the One Shot® transformation protocol provided on page 20.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

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Troubleshooting, continued

Expression

The table below lists some potential problems and possible solutions that may help you troubleshoot your expression experiment. To help evaluate your results, we recommend including the expression control supplied with kit in your experiment.

Problem	Reason	Solution
No expression of recombinant protein	Gene of interest not in frame with the epitope tag	Sequence your construct to verify if the insert is in frame with the epitope tag. If not in frame, redesign your PCR primers.
	Incorrect antibody used for detection	Use an antibody to your protein or one of the antibodies listed on page xii, as appropriate
Low expression	Plasmid instability observed when using ampicillin for selection	Substitute carbenicillin for ampicillin in your transformation and expression experiments (see page 25).
	Toxic gene **Note:** Evidence of toxicity includes loss of plasmid or slow growth relative to the control.	• Add 1% glucose to the bacterial culture medium during transformation and expression. • Transform BL21 Star™(DE3) cells using the protocol on page 26, then perform the expression by growing cells at room temperature rather than 37°C for 24-48 hours. • Transform your expression construct into a pLysS-containing strain (*e.g.* BL21 Star™(DE3)pLysS). • Transform your expression construct into an *E. coli* strain in which expression of T7 RNA polymerase is tightly regulated (*e.g.* BL21-AI™ available from Invitrogen; see our Web site for more information). • Infect TOP10F′ (or other suitable F′ episome-containing host strain) with M13 or lambda phage expressing T7 RNA polymerase.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing the appropriate antibiotic (see page 54 for a recipe).

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Thermostable polymerase (1-2.5 units/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR produce used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pET TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pET TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

Incubate at room temperature for 5 minutes and place on ice.

Transform 3 l of each reaction into separate vials of One Shot® TOP10 cells (page 20).

Spread 100-200 l of each transformation mix onto LB plates containing the appropriate antibiotic. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.

Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pET100/D-TOPO®	*Hin*d III	Correct orientation: 838, 5674 Reverse orientation: 500, 6012 Empty vector: 5764
pET101/D-TOPO®	*Cla* I	Correct orientation: 599, 5896 Reverse orientation: 929, 5566 Empty vector: 5753
pET102/D-TOPO®	*Pst* I	Correct orientation: 1990, 5065 Reverse orientation: 1484, 5571 Empty vector: 6315

continued on next page

Performing the Control Reactions, continued

Analysis of Results, continued

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pET151/D-TOPO®	*Eco*R I and *Sst* I	Correct orientation: 653, 5861 Reverse orientation: 101, 6413 Empty vector: 5760
pET200/D-TOPO®	*Hin*d III	Correct orientation: 845, 5646 Reverse orientation: 511, 5980 Empty vector: 5741

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation.

Relatively few colonies should be produced in the vector-only reaction.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 20. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are provided below.

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit (page xi) allows you to rapidly purify PCR products from regular agarose gels.

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment.
3. Weigh the gel slice.
4. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
   - For ≤2% agarose gels, place up to 400 mg gel into a sterile, 1.5-ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-ml polypropylene tubes and add 60 µl Gel Solubilization Buffer (GS1) for every 10 mg of gel.
5. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate for an **additional** 5 minutes.
6. Preheat an aliquot of TE Buffer (TE) to 65-70°C
7. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from Step 5,above onto the column. Use 1 column per 400 mg agarose.
8. Centrifuge at >12,000 x g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
9. **Optional:** Add 500 µl Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 x g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
10. Add 700 µl Wash Buffer (W9) with ethanol (add 96–100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate at room temperature for 5 minutes. Centrifuge at >12,000 x g for 1 minute. Discard flow-through.
11. Centrifuge the column at >12,000 x g for 1 minute to remove any residual buffer. Place the column into a 1.5 ml Recovery Tube.
12. Add 50 µl **warm** (65-70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
13. Centrifuge at >12,000 x g for 2 minutes. *The Recovery Tube contains the purified DNA.* Store DNA at –20°C. Discard the column.
14. Use 4 µl of the purified DNA for the TOPO® Cloning reaction.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in dilution of your PCR product and a potential loss of cloning efficiency. Use only chemically competent cells for transformation.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 18.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the method on page 20.

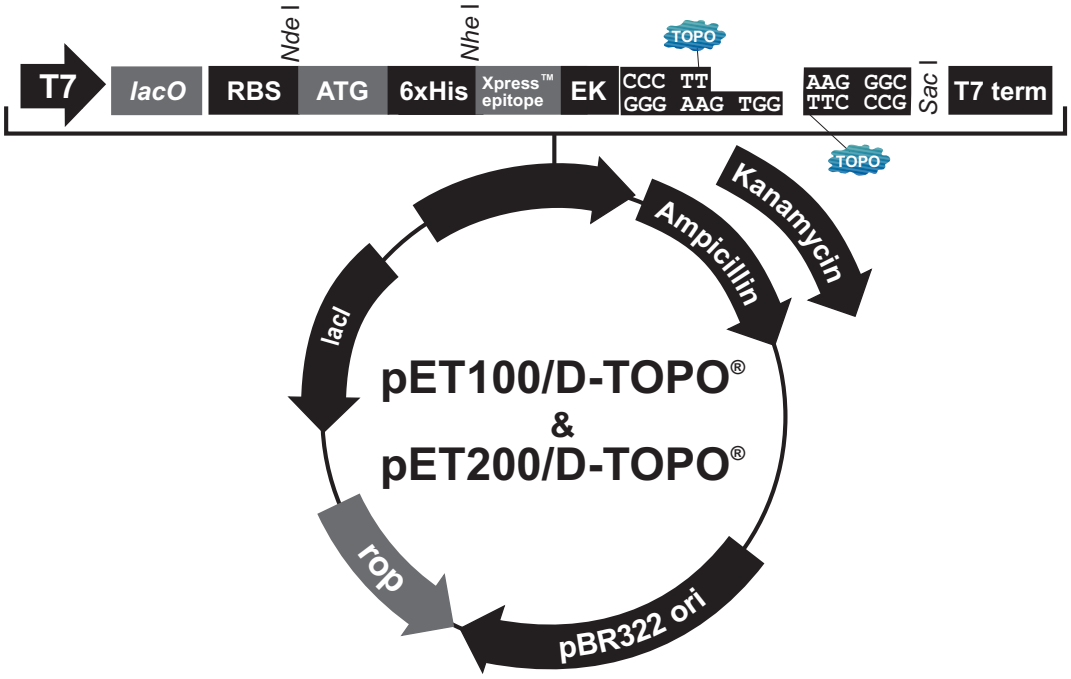
Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Map and Features of pET100/D-TOPO® and pET200/D-TOPO®

Map

The figure below shows the features of the pET100/D-TOPO® (5764 bp) and pET200/D-TOPO® (5741 bp) vectors. **The complete sequence of each vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**



	pET100/D-TOPO®	pET200/D-TOPO®
T7 promoter	209-225	209-225
T7 promoter/priming site	209-228	209-228
lac operator (lacO)	228-252	228-252
Ribosome binding site (RBS)	282-288	282-288
Initiation ATG	297-299	297-299
Polyhistidine (6xHis) region	309-326	309-326
Xpress™ epitope	366-389	366-389
EK recognition site	375-389	375-389
TOPO® Cloning site (directional)	396-409	396-409
T7 reverse priming site	466-485	466-485
T7 transcription termination region	427-555	427-555
bla promoter	856-954	856-954
Ampicillin (*bla*) resistance gene	955-1815	---
Kanamycin resistance gene	---	955-1749
pBR322 origin	2022-2757	1969-2610
ROP ORF (complementary strand)	3001-3192	2978-3169
lacI ORF (complementary strand)	4507-5595	4481-5572

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Map and Features of pET100/D-TOPO® and pET200/D-TOPO®, continued

Features of pET100/D-TOPO® and pET200/D-TOPO®

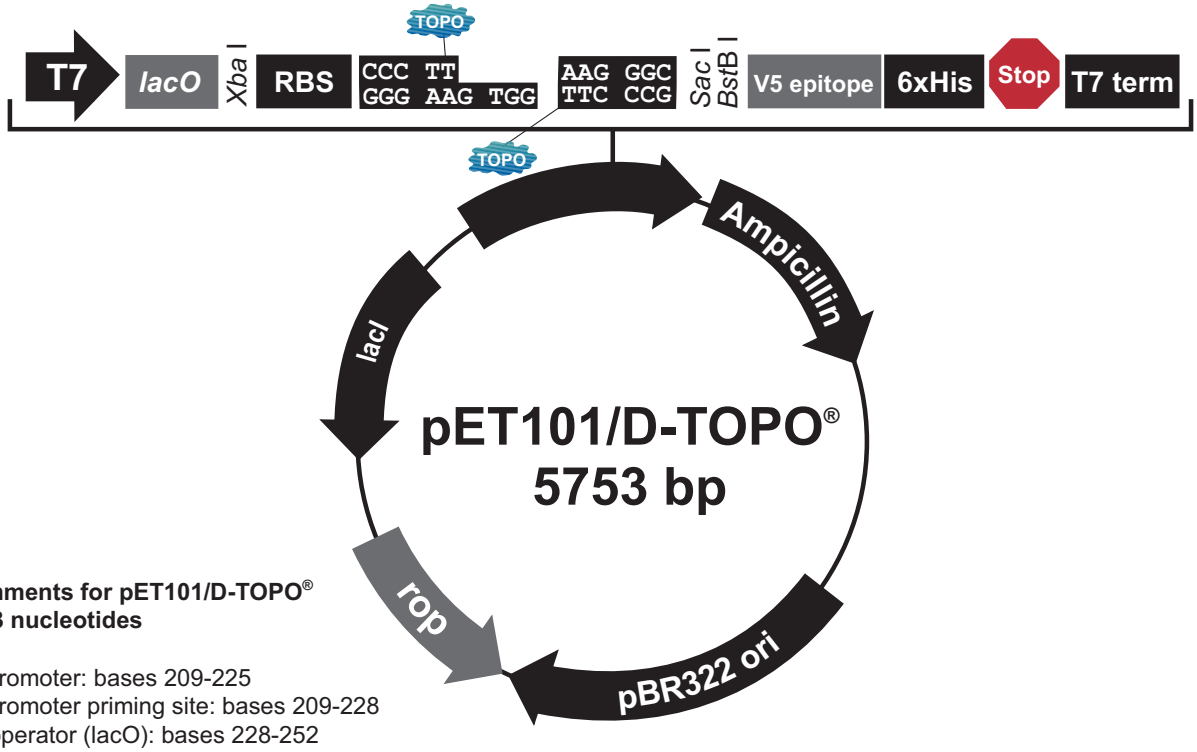
The pET100/D-TOPO® (5764 bp) and pET200/D-TOPO® (5741 bp) vectors contain the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the TOPO® Cloning site for efficient translation of PCR product.
N-terminal 6xHis tag	Permits purification of recombinant fusion protein on metal-chelating resin (*i.e.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-HisG Antibodies.
Xpress™ epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys)	Allows detection of the fusion protein by the Anti-Xpress™ Antibodies.
Enterokinase (EK) recognition site (Asp-Asp-Asp-Asp-Lys)	Allows removal of the N-terminal tag from your recombinant protein using an enterokinase such as EKMax™.
TOPO® Cloning site (directional)	Permits rapid cloning of your PCR product for expression in *E. coli.*
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from bacteriophage T7 which permits efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase) (pET100/D-TOPO® only)	Allows selection of the plasmid in *E. coli*.
Kanamycin resistance gene (pET200/D-TOPO® only)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Permits replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

Map and Features of pET101/D-TOPO®

pET101/D-TOPO® Map

The figure below shows the features of the pET101/D-TOPO® (5753 bp) vector. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**



Comments for pET101/D-TOPO®
5753 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288, 292-296
TOPO® cloning site (directional): bases 297-310
V5 epitope: bases 333-374
Polyhistidine (6xHis) region: bases 384-401
T7 reverse priming site: bases 455-474
T7 transcription termination region: bases 416-544
bla promoter: bases 845-943
Ampicillin (*bla*) resistance gene (ORF): bases 944-1804
pBR322 origin: bases 1949-2622
ROP ORF: bases 2990-3181 (complementary strand)
lacI ORF: bases 4493-5584 (complementary strand)

continued on next page

Map and Features of pET101/D-TOPO®, continued

Features of pET101/D-TOPO®

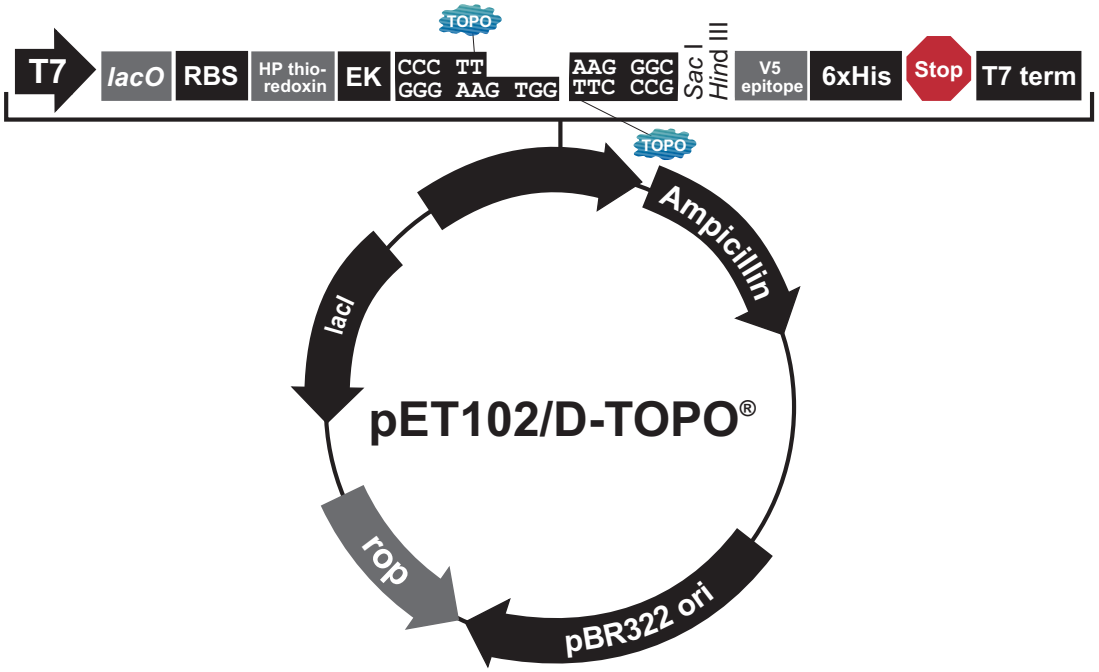
The pET101/D-TOPO® (5753 bp) vector contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the TOPO® Cloning site for efficient translation of PCR product.
TOPO® Cloning site (directional)	Permits rapid cloning of your PCR product for expression in *E. coli.*
C-terminal V5 epitope tag (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991).
C-terminal 6xHis tag	Permits purification of recombinant fusion protein on metal-chelating resins (*i.e.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-His(C-term) Antibodies (Lindner *et al.*, 1997).
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from T7 bacteriophage which permits efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Permits replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

Map and Features of pET102/D-TOPO®

pET102/D-TOPO® Map

The figure below shows the features of the pET102/D-TOPO® (6315 bp) vector. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**



	pET102/D-TOPO®
T7 promoter	209-225
T7 promoter/priming site	209-228
lac operator (lacO)	228-252
Ribosome binding site (RBS)	282-288
His-patch (HP) thioredoxin ORF	298-627
TrxFus forward priming site	607-624
EK recognition site	643-657
TOPO® Cloning site (directional)	670-683
V5 epitope	700-741
Polyhistidine (6xHis) region	751-768
T7 reverse priming site	822-841
T7 transcription termination region	783-911
bla promoter	1407-1505
Ampicillin (*bla*) resistance gene	1506-2366
pBR322 origin	2511-3184
ROP ORF (complementary strand)	3552-3743
lacI ORF (complementary strand)	5055-6146

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Map and Features of pET102/D-TOPO®, continued

Features of pET102/D-TOPO®

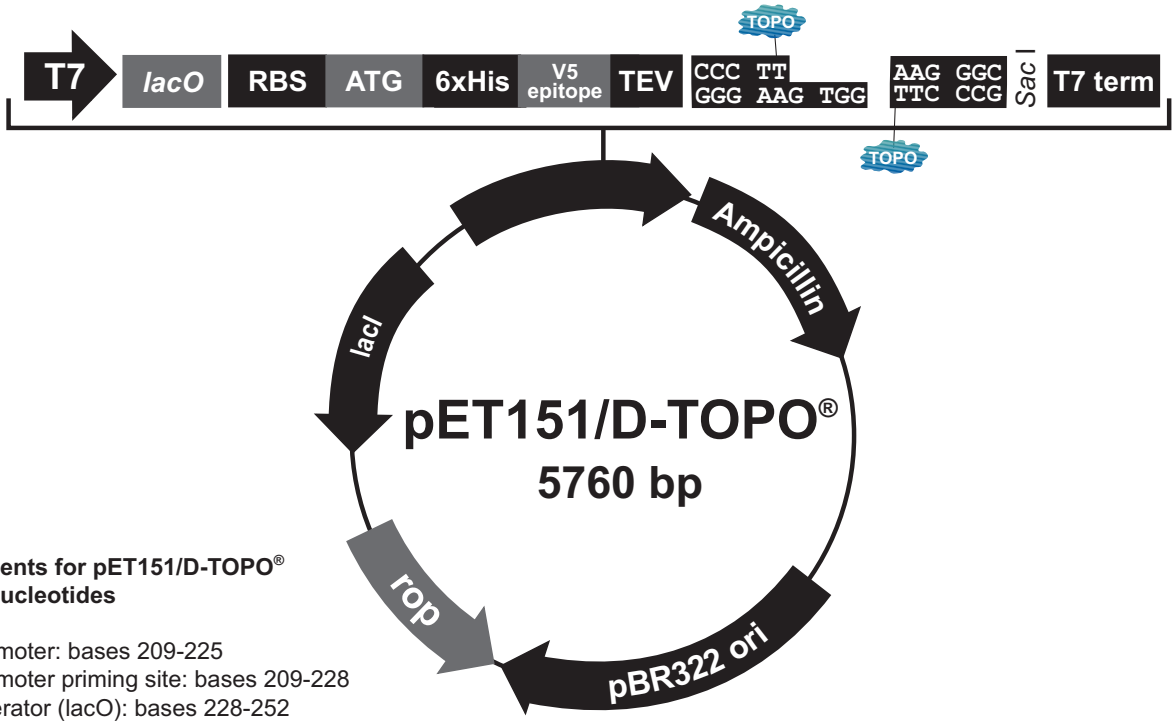
The pET102/D-TOPO® (6315 bp) vector contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the TOPO® Cloning site for efficient translation of PCR product.
His-Patch (HP)-thioredoxin	Provides an efficient fusion partner for translation of the fusion protein.
TrxFus forward priming site	Permits sequencing of the insert in the sense orientation.
Enterokinase (EK) recognition site (Asp-Asp-Asp-Asp-Lys)	Allows removal of the N-terminal tag from your protein using an enterokinase such as EKMax™.
TOPO® Cloning site (directional)	Permits rapid cloning of your PCR product.
C-terminal V5 epitope tag	Allows detection of the fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991).
C-terminal 6xHis tag	Permits purification of recombinant fusion protein on metal-chelating resins (*i.e.* ProBond™ or Ni-NTA). In addition, allows detection of the recombinant protein with the Anti-His(C-term) Antibodies (Lindner *et al.*, 1997).
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from T7 bacteriophage which permits efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase) (pET102/D-TOPO® only)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Permits replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

Map and Features of pET151/D-TOPO®

Map of pET151/D-TOPO®

The figure below shows the features of the pET151/D-TOPO® (5760 bp) vector. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**



Comments for pET151/D-TOPO®
5760 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-289
Initiation ATG: bases 297-299
Polyhistidine (6xHis) region: bases 300-317
V5 epitope: bases 318-359
TEV recognition site: bases 360-380
TOPO® cloning site (directional): bases 387-400
T7 reverse priming site: bases 455-474
T7 transcription termination region: bases 416-544
bla promoter: bases 849-947
Ampicillin (*bla*) resistance gene: bases 948-1808
pBR322 origin: bases 1953-2626
ROP ORF: bases 2997-3188 (complementary strand)
lacI ORF: bases 4500-5612 (complementary strand)

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Map and Features of pET151/D-TOPO®, continued

Features of pET151/D-TOPO®

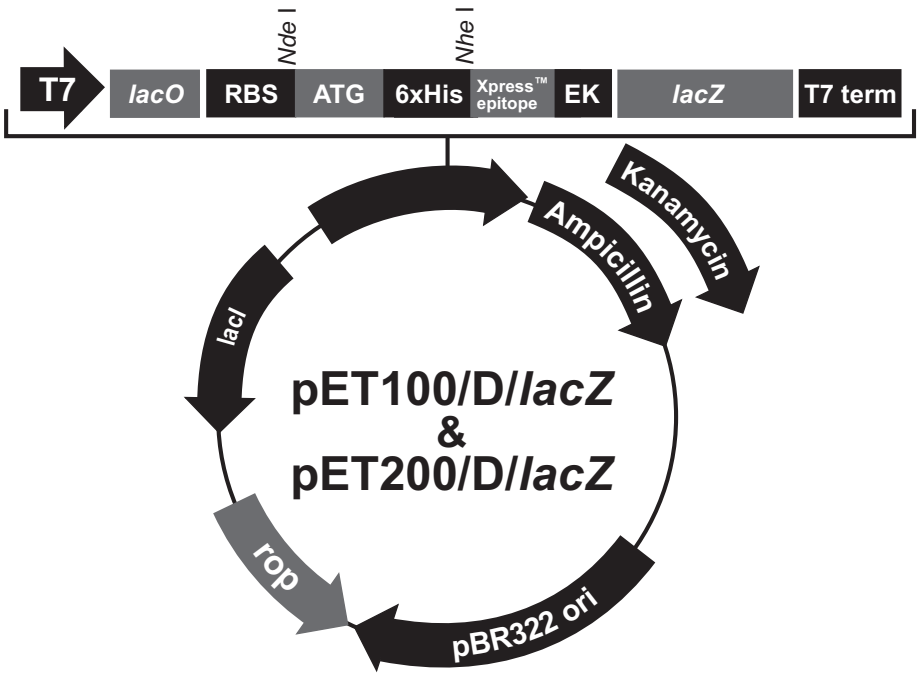
The pET151/D-TOPO® (5760 bp) vector contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the TOPO® Cloning site for efficient translation of PCR product.
N-terminal 6xHis tag	Permits purification of recombinant fusion protein on metal-chelating resin (*i.e.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-HisG Antibodies.
V5 epitope	Allows detection of the fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991).
TEV recognition site	Allows removal of the N-terminal tag from your recombinant protein using TEV protease (Carrington & Dougherty, 1988; Dougherty *et al.*, 1988).
TOPO® Cloning site (directional)	Permits rapid cloning of your PCR product for expression in *E. coli.*
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from bacteriophage T7 which permits efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Permits replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

Map of pET100/D/*lacZ* and pET200/D/*lacZ*

Description

pET100/D/*lacZ* (8836 bp) and pET200/D/*lacZ* (8813 bp) are vectors containing a *lacZ* gene that has been directionally TOPO® Cloned into pET100/D-TOPO® and pET200/D-TOPO®, respectively. In each vector, the *lacZ* gene is cloned in frame with the N-terminal peptide containing the Xpress™ epitope and the 6xHis tag. The size of the β-galactosidase fusion protein is approximately 121 kDa. **The nucleotide sequence of each vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**

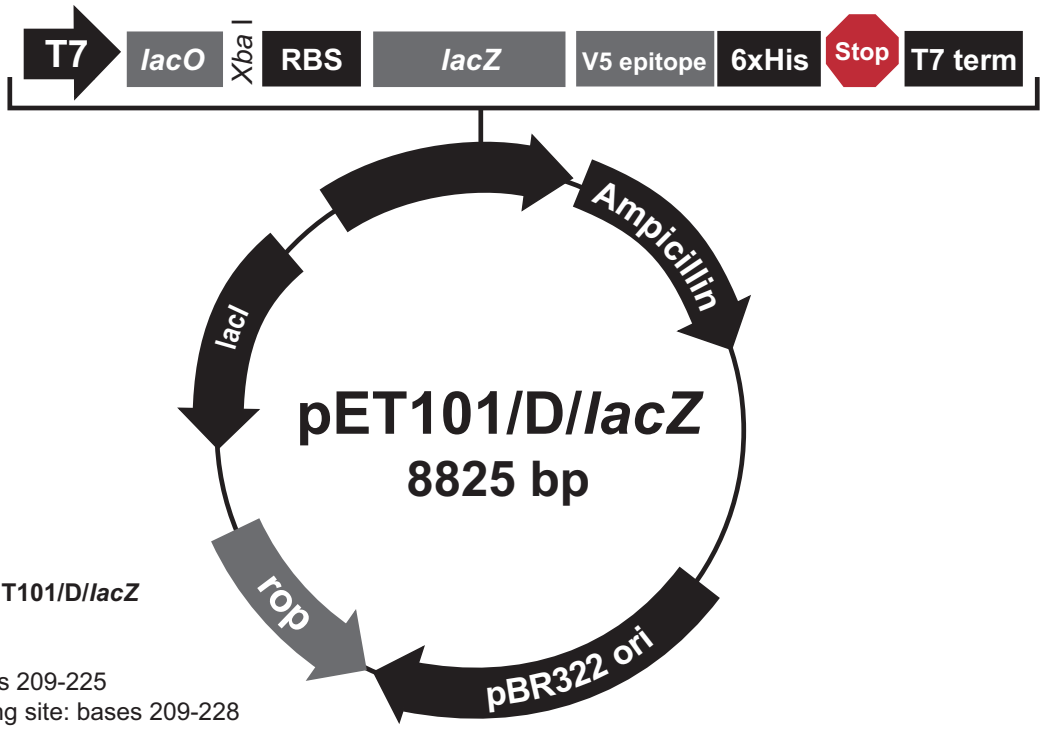


	pET100/D/*lacZ*	pET200/D/*lacZ*
T7 promoter	209-225	209-225
T7 promoter/priming site	209-228	209-228
lac operator (lacO)	228-252	228-252
Ribosome binding site (RBS)	282-288	282-288
Initiation ATG	297-299	297-299
Polyhistidine (6xHis) region	309-326	309-326
Xpress™ epitope	366-389	366-389
EK recognition site	375-389	375-389
lacZ ORF	405-3476	405-3476
T7 reverse priming site	3538-3557	3538-3557
T7 transcription termination region	3499-3627	3499-3627
bla promoter	3928-4026	3928-4026
Ampicillin (*bla*) resistance gene	4027-4887	---
Kanamycin resistance gene	---	4027-4821
pBR322 origin	5032-5705	5009-5682
ROP ORF (complementary strand)	6073-6264	6050-6241
lacI ORF (complementary strand)	7576-8667	7553-8644

Map of pET101/D/*lacZ*

Description

The pET101/D/*lacZ* (8825 bp) vector contains a *lacZ* gene that has been directionally TOPO® Cloned into pET101/D-TOPO® in frame with the C-terminal peptide containing the V5 epitope and the 6xHis tag. The size of the β-galactosidase fusion protein is approximately 120 kDa. **The nucleotide sequence of the vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**



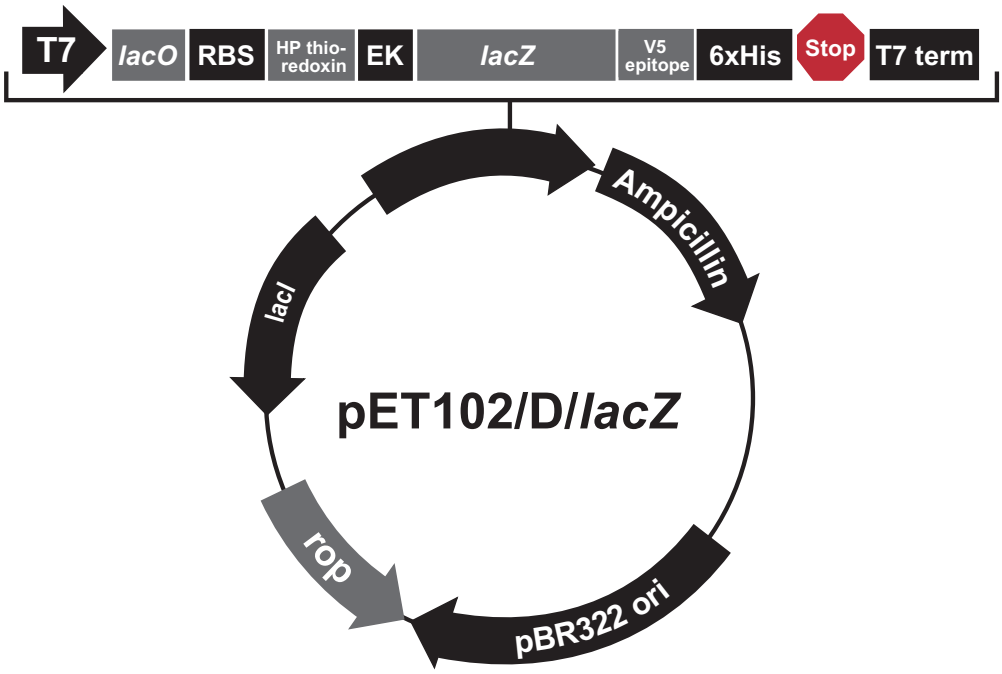
Comments for pET101/D/*lacZ*
8825 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288, 292-296
lacZ ORF: bases 306-3362
V5 epitope: bases 3405-3446
Polyhistidine (6xHis) region: bases 3456-3473
T7 reverse priming site: bases 3527-3546
T7 transcription termination region: bases 3488-3616
bla promoter: bases 3917-4015
Ampicillin (*bla*) resistance gene (ORF): bases 4016-4876
pBR322 origin: bases 5021-5694
ROP ORF: bases 6062-6253 (complementary strand)
lacI ORF: bases 7565-8656 (complementary strand)

Map of pET102/D/*lacZ*

Description

pET102/D/*lacZ* (9384 bp) is a vector containing a *lacZ* gene that has been directionally TOPO® Cloned into pET102/D-TOPO®. The *lacZ* gene is cloned in frame with the N-terminal HP thioredoxin and the C-terminal peptide tag. The size of the β-galactosidase fusion protein is approximately 133 kDa. **The nucleotide sequence of the vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**

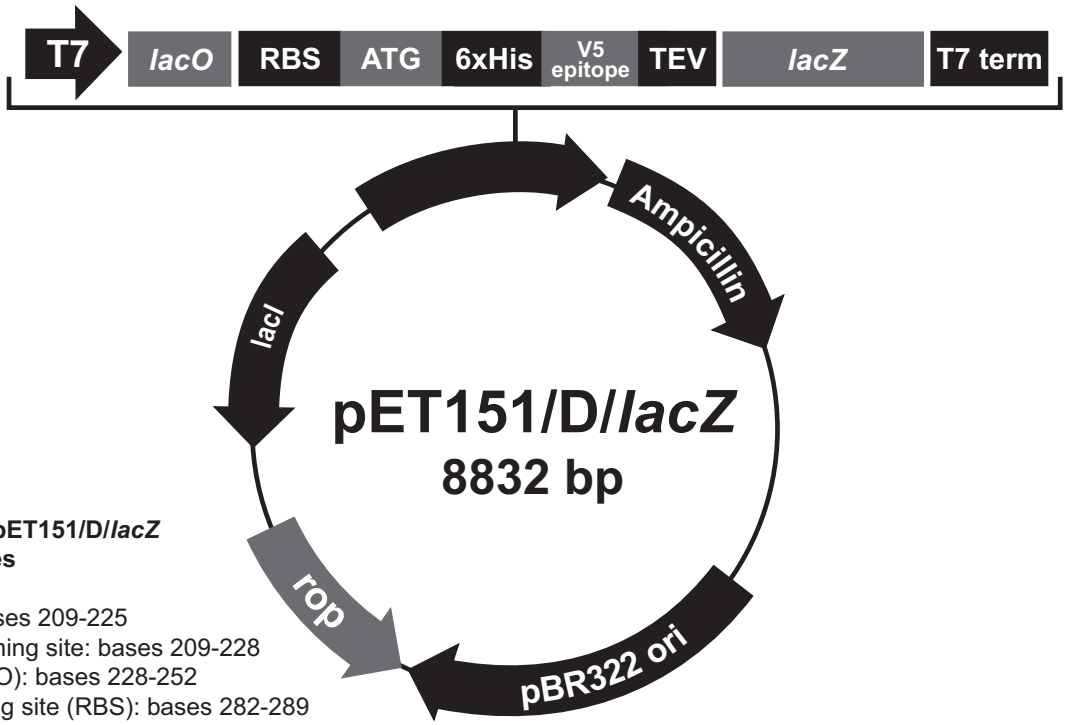


	pET102/D/*lacZ*
T7 promoter	209-225
T7 promoter/priming site	209-228
lac operator (lacO)	228-252
Ribosome binding site (RBS)	282-288
His-patch (HP) thioredoxin ORF	298-627
TrxFus forward priming site	607-624
EK recognition site	643-657
lacZ ORF	679-3732
V5 epitope	3769-3810
Polyhistidine (6xHis) region	3820-3837
T7 reverse priming site	3891-3910
T7 transcription termination region	3852-3980
bla promoter	4476-4574
Ampicillin (*bla*) resistance gene	4575-5435
pBR322 origin	5580-6253
ROP ORF (complementary strand)	6621-6812
lacI ORF (complementary strand)	8124-9215

Map of pET151/D/*lacZ*

Description

The pET151/D/*lacZ* (8832 bp) vector contains a *lacZ* gene that has been directionally TOPO® Cloned into pET151/D-TOPO® in frame with the N-terminal peptide containing the V5 epitope and the 6xHis tag. The size of the β-galactosidase fusion protein is approximately 120 kDa. **The nucleotide sequence of the vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Service (see page 56).**



Comments for pET151/D/*lacZ*
8832 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-289
Initiation ATG: bases 297-299
Polyhistidine (6xHis) region: bases 300-317
V5 epitope: bases 318-359
TEV recognition site: bases 360-380
lacZ ORF: bases 396-3467
T7 reverse priming site: bases 3527-3546
T7 transcription termination region: bases 3488-3616
bla promoter: bases 3921-4019
Ampicillin (*bla*) resistance gene: bases 4020-4880
pBR322 origin: bases 5025-5698
ROP ORF: bases 6069-6260 (complementary strand)
lacI ORF: bases 7572-8684 (complementary strand)

Recipes

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

S.O.B. Medium (with Antibiotic)

2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$. You may also add antibiotic, if needed.
5. Store at +4°C. **Medium is stable for only 1-2 weeks.**

continued on next page

Recipes, continued

Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.

For 100 ml, dissolve the following reagents in 90 ml of deionized water:

- 0.3 ml KH_2PO_4
- 4.7 ml K_2HPO_4
- 2.3 g NaCl
- 0.75 g KCl
- 10 ml glycerol
- 0.5 ml Triton X-100
- 68 mg imidazole

Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.

Store at +4°C.

2X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2.0 ml
β-mercaptoethanol	0.4 ml
Bromophenol Blue	0.02 g
SDS	0.4 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

1X SDS-PAGE Sample Buffer

Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	1.25 ml
Glycerol (100%)	1.0 ml
β-mercaptoethanol	0.2 ml
Bromophenol Blue	0.01 g
SDS	0.2 g

Bring the volume to 10 ml with sterile water.

Aliquot and freeze at -20°C until needed.

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the Champion™ pET Directional TOPO® Expression Kits.

Vectors

Restriction analysis with the enzymes below is performed on the parent vector of each pET TOPO® vector to confirm its identity. In each case, the supercoiled vector is qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

TOPO® Cloning Efficiency

Once the supercoiled vectors have been adapted with topoisomerase I, they are lot-qualified using the control reagents included in the kit. Under conditions described on pages 37-38, a 750 bp control PCR product is amplified using a forward primer containing CACC at its 5′ end and a reverse primer. The product is TOPO® Cloned into the pET TOPO® vector and subsequently transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency. Forty transformants are characterized by restriction digest. Of the transformants characterized, greater than 90% should contain an insert in the correct orientation.

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Chemically Competent *E. coli*

All competent cells are tested for transformation efficiency using the control plasmid included in the One Shot® kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be:

- Greater than 1 x 10^9 cfu/µg plasmid DNA for TOP10
- Greater than 1 x 10^8 cfu/µg plasmid DNA for BL21 Star™(DE3)

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and absence of phage contamination.

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